NIH RELAIS Document Delivery

NIH-10336041

RIEDT

NIH -- W1 ME9616JM

THOMAS RIED
Bldg. 9 1N105 NCI
9 Memorial Drive
Bethesda, MD 20892

ATTN:
PHONE: 301-594-3118
FAX: 301-402-1204
E-MAIL:

SUBMITTED: 2002-11-06 10:59:34
PRINTED: 2002-11-08 11:03:04
REQUEST NO.:NIH-10336041
SENT VIA: LOAN DOC
8686829

NIH Fiche to Paper Journal

TITLE: METHODS IN MOLECULAR MEDICINE
PUBLISHER/PLACE: Humana Press, Totowa, N.J. :
VOLUME/ISSUE/PAGES:2002;68():29-44 29-44
DATE: 2002
AUTHOR OF ARTICLE: Hilgenfeld E; Montagna C; Padilla-Nash H; Stapleton L; Hesel
TITLE OF ARTICLE: Spectral karyotyping in cancer cytogenetics.
ISSN: [NOT AVAI
OTHER NOS/LETTERS: Library does NOT report holding title
101123138
11901510
SOURCE: PubMed
CALL NUMBER: W1 ME9616JM
REQUESTER INFO: RIEDT
DELIVERY: E-mail: buddyc@mail.nih.gov
REPLY: Mail:

3

Spectral Karyotyping in Cancer Cytogenetics

Eva Hilgenfeld, Cristina Montagna, Hesed Padilla-Nash, Linda Stapleton, Kerstin Heselmeyer-Haddad, and Thomas Ried

1. Introduction

Cancer is a genetic disease. Gene mutations are not only responsible for rare hereditary forms of human cancer, but for the sporadic forms of human malignancies as well. Many of these specific genetic defects in cancer cells can be visualized as chromosomal aberrations. Conventional cytogenetic analysis of metaphase chromosomes from human malignancies is a first screening step to identify chromosomal aberrations. Since the introduction of chromosome banding techniques in 1970 by Caspersson et al. *(1)*, significant knowledge of chromosomal aberrations especially in hematologic malignancies as well as sarcomas has been gained. In these malignancies, specific balanced translocations were identified and have led to the cloning of the genes involved at many breakpoints. These aberrations have proven to be of significant etiologic, diagnostic, prognostic, as well as therapeutic relevance, especially in leukemias. While cytogenetic analyses have been exceedingly valuable for the description of chromosomal abnormalities in hematologic malignancies and in sarcomas, epithelial cancers were more difficult to study. This is owing, in part, not only to the accessibility of malignant cells and subsequently metaphases for cytogenetic analysis in leukemias, but also to the nature of reciprocal translocations, which provided more immediate entry points for positional cloning efforts.

Although cytogenetic methodologies for the analysis of solid tumor specimens have improved, the difficulty in obtaining good-quality metaphase chromosomes remains *(2)*. The interpretation of cytogenetic abnormalities in epithelial cancers is further confounded by the often vast number and complex nature of chromosomal aberrations in these tumors. Still, recurrent aberrations

From: *Methods in Molecular Medicine, vol. 68: Molecular Analysis of Cancer*
Edited by: J. Boultwood and C. Fidler

and recurrent chromosomal imbalances have been identified, but their clinical relevance is less firmly established *(2–4)*.

Some of the limitations of chromosome banding techniques were overcome by the introduction of molecular cytogenetic techniques such as fluorescence *in situ* hybridization (FISH) with chromosome-painting probes and comparative genomic hybridization (CGH) *(5–7)*. For example, in hematologic malignancies, the t(12;21)(p13;q22) was detected by chromosome painting, because the telomeric regions involved in this translocation are indistinguishable by banding techniques *(8)*. The 12;21 translocation was ascertained to be the most common chromosomal aberration in pediatric B-ALL and has been associated with a favorable prognosis *(9)*. In solid tumors, the application of CGH has led to the identification of recurring patterns of genomic imbalances, both for different tumors and for distinct tumor stages *(10,11)*.

Herein we focus on recently introduced molecular cytogenetic screening techniques that allow one to visualize all human metaphase chromosomes in specific colors.

1.1. Methodology of SKY

Two alternative techniques were developed for color karyotyping: combinatorial multifluor FISH (M-FISH) and spectral karyotyping (SKY) *(12,13)*. Whereas M-FISH employs a conventional imaging approach requiring multiple exposures through a series of single bandpass filters *(12)*, SKY utilizes a novel approach by combining Fourier spectroscopy with epifluorescence microscopy and charge-coupled device (CCD)-imaging, thereby measuring the entire spectrum at all points in a single exposure *(13,14)*.

For SKY, 24 differentially labeled chromosome libraries are produced by amplifying flow-sorted chromosomes utilizing a degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) *(15)*. Subsequently, the probes are labeled through the incorporation of either haptenized (biotin and digoxigenin) or directly labeled nucleotides, again via PCR. The use of five fluorochromes, either alone or in combination, allows one to discern up to 31 targets simultaneously. The generated chromosome-specific probes are pooled, precipitated with an excess of Cot-1 DNA to suppress repetitive sequences (suppression hybridization), and hybridized onto metaphase chromosomes. The use of an epifluorescence microscope equipped with a single, custom-designed triple bandpass filter allows for the simultaneous excitation of all fluorochromes as well as measurement of the entire emission spectrum of one metaphase in a single exposure. The emitted light from each point of the metaphase is passed through the collection optics and subsequently the Sagnac interferometer, where an optical path difference is created. The resulting interferogram is measured for every pixel of the CCD camera and, using Fourier

transformation, is converted to spectral information. The spectral image can be displayed first in RGB colors (obtained by assigning red, green, and blue to specific sections of the emission spectrum) to evaluate the quality of the hybridization (i.e., homogeneity). Every pixel with the same spectral information is subsequently assigned a pseudo-color allowing the spectral classification of all chromosomes *(14)*. **Figure 1A–C** shows a metaphase of the human bladder carcinoma cell line HT1197 displayed in the RGB colors with the accompanying 4,6-diamidino-2-phenylindole (DAPI)-image, and the SKY classification colors.

1.2. Advantages and Limitations

SKY, which is a screening tool, combines the respective advantages of chromosome banding techniques with the advantages of FISH. SKY is especially useful for the detection of interchromosomal structural aberrations that lead to color changes of the aberrant chromosome, such as translocations and insertions. It therefore facilitates the identification of cryptic translocations as well as the clarification of complex aberrations. In addition, SKY assists in the identification of material not recognizable by banding techniques such as marker and ring chromosomes. Other aberrations important in tumor cytogenetics such as double minute chromosomes as well as homogeneously staining regions, which are aberrations that harbor amplified DNA sequences, can be better resolved and contribute to the identification of critical oncogenes. Since its introduction, the value of SKY for use in cancer cytogenetics has been amply demonstrated (for a review, *see* **ref. *16***).

Limitations of the technique pertain to intrachromosomal changes, such as para- or pericentric inversions as well as small deletions or duplications that do not lead to a color change or change in size of the respective aberrant chromosome, which then can be identified more readily in conjunction with the inverted DAPI image or other banding techniques. However, very small marker chromosomes or double minute chromosomes cannot in all instances be classified unambiguously, perhaps owing to the fact that their euchromatin content is low. Therefore, for a comprehensive analysis of tumor metaphases, a combination of molecular cytogenetic methods and banding techniques is advocated.

1.3. Applications of SKY

The usefulness of SKY for cancer cytogenetics, of hematologic malignancies as well as solid tumors, has been shown (for a review, *see* **ref. *16***). Although the difficulty in obtaining good metaphase chromosomes from primary solid tumors remains, SKY analysis of the often complex karyotypes contributes to a more comprehensive cytogenetic analysis and might assist in the identification of stage-specific aberrations *(17–19)*.

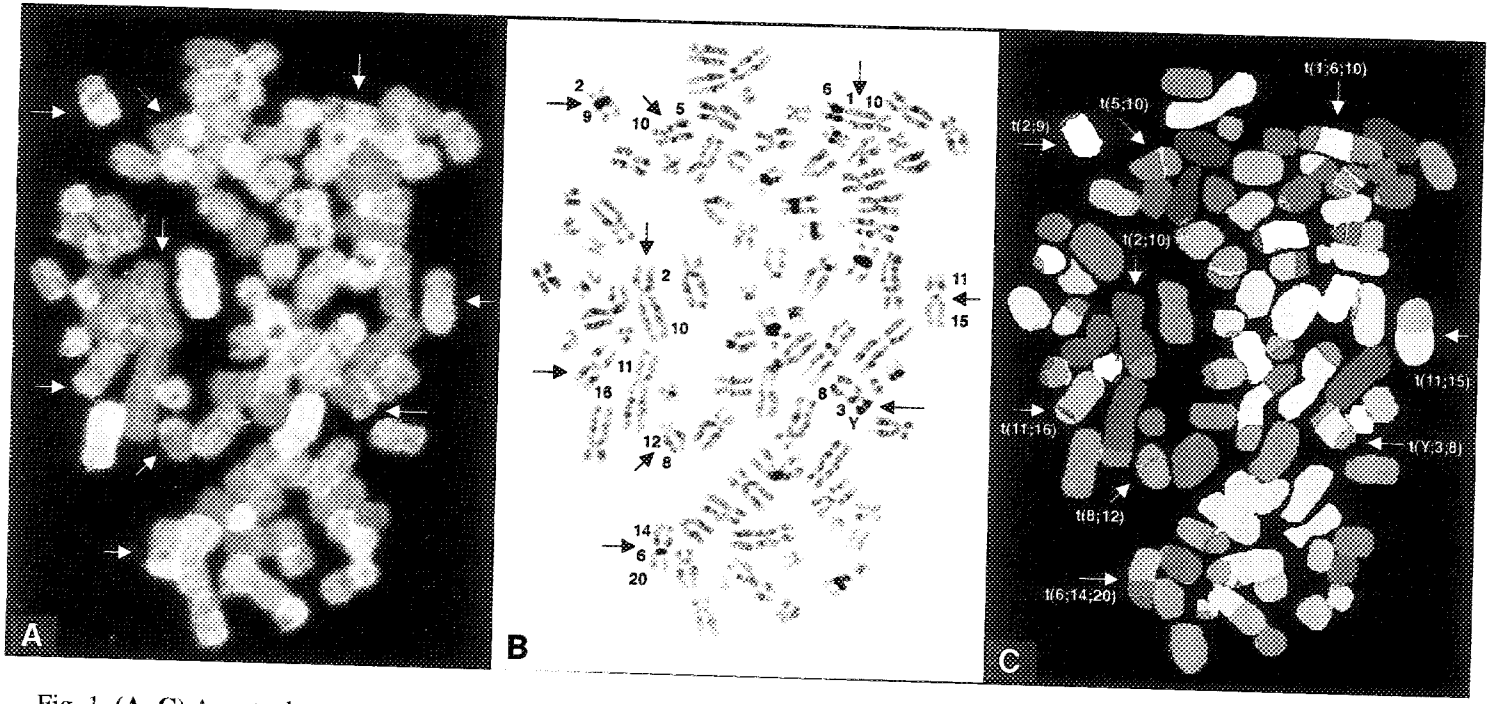

Fig. 1. **(A–C)** A metaphase spread of the human bladder carcinoma cell line HT 1197 in the RGB-colors (A), the corresponding inverted DAPI image (B), and the SKY classification colors (C). Some, but not all aberrations present within this complex karyotype are marked by arrows.

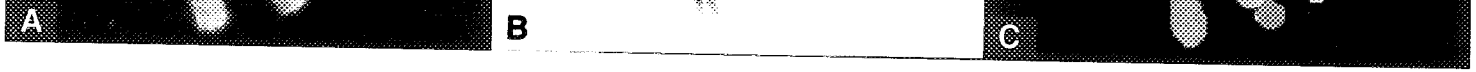

Fig. 1. **(A–C)** A metaphase spread of the human bladder carcinoma cell line HT 1197 in the RGB-colors (A), the corresponding inverted DAPI image (B), and the SKY classification colors (C). Some, but not all aberrations present within this complex karyotype are marked by arrows.

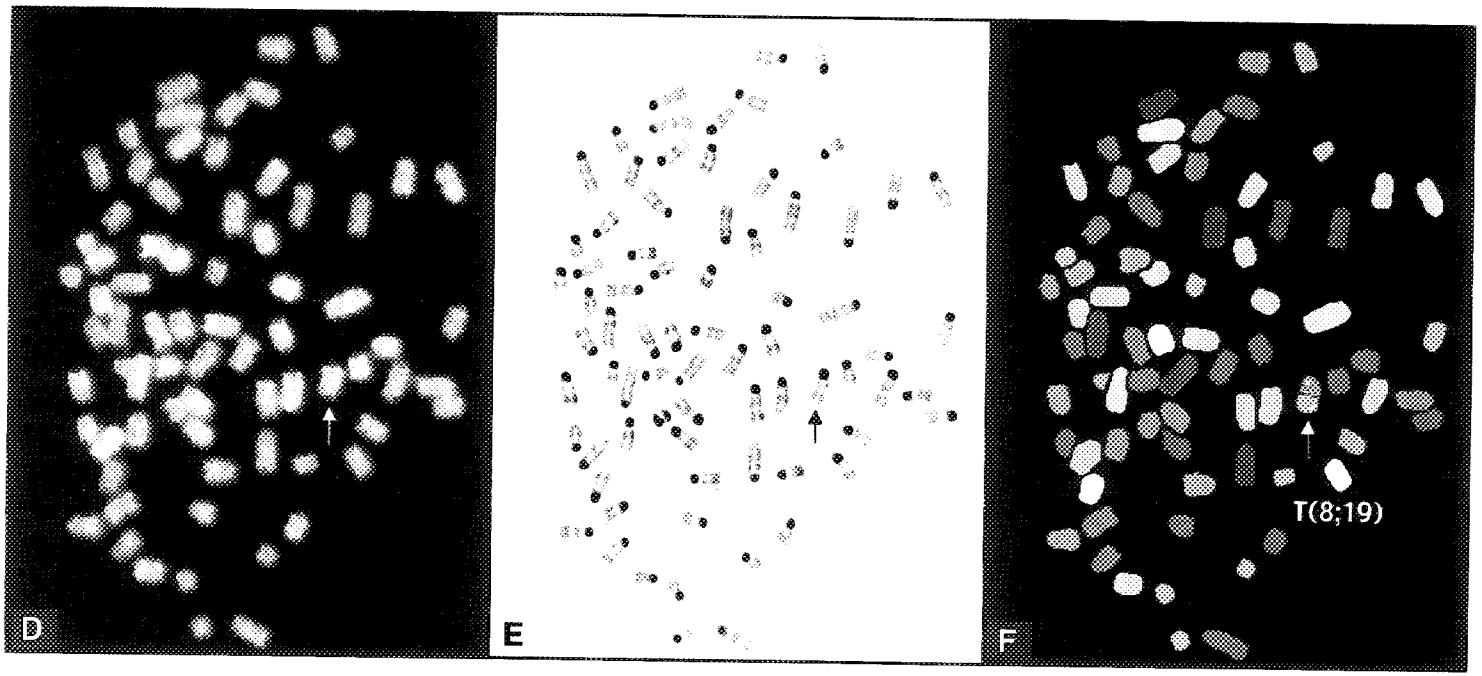

Fig. 1. **(D–F)** Depicts a metaphase prepared from a cell line derived from a mouse mammary tumor. (D) Shows the metaphase in RGB colors, (E) in the corresponding inverted DAPI image, and (F) in the SKY classification colors. The T(8;19) is identified in all three images.

In contrast to the common assumption that cytogenetic changes in cell lines are frequently the result of culture artifacts, the molecular cytogenetic analysis of tumor cell lines showed that the karyotype is surprisingly stable after years of culturing *(20–22)*. Furthermore, results of the SKY analysis of pancreatic cell lines correlated well with those of the CGH analysis of primary tumors *(21)*. In contrast to CGH, SKY can detect the specific type of aberrations that result in chromosomal gains, the amplification of putative oncogenes (e.g., duplications, double minute chromosomes, homogeneously staining regions, jumping translocations), as well as loss of chromosomal material that may harbor tumor suppressor genes [e.g., deletions, isochromosomes such as i(17)(q10)]. Therefore, SKY analysis might not only contribute to the comprehensive analysis of complex aberrations, but also to our understanding of the mechanisms leading to these changes *(23)*.

Mouse models of human disease become more and more important for our understanding of malignancies. As they often can be studied at earlier stages of carcinogenesis, they hold the promise for identification of tumor-initiating events as well as the dissection of genetic events responsible for tumor progression. Nevertheless, the analysis of mouse chromosomes is challenging because mouse chromosomes are all acrocentric and of similar size. The adaptation of SKY to the mouse karyotype by Liyanage et al. *(24,27)* has proven to be a very valuable tool in the analysis of several mouse models *(24–28)*. Comprehensive SKY analyses have shown that chromosomal aberrations in the aforementioned mouse tumors are similar to the changes in the respective human tumors, thereby validating these models. **Figure 1D–F** displays a mouse metaphase in the RGB, inverted DAPI, and SKY classification colors.

1.4. Further Tools and Future Goals

To collect the increasing amount of emerging SKY data and to expedite the identification of new recurrent tumor or tumor stage-specific aberrations, a database has been developed (www.ncbi.nlm.nih.gov/sky/skyweb.cgi).

This database is linked to the Cancer Chromosome Aberration Project (CCAP) (www.ncbi.nlm.nih.gov/CCAP), which integrates the physical and sequence maps with the cytogenetic map of the human genome *(29)*. This project provides STS-tagged and sequenced BAC clones for the entire human genome, whose cytogenetic location has been determined by high-resolution FISH mapping with a resolution of 1 to 2 Mb. CCAP facilitates the high-resolution mapping of chromosomal breakpoints and the subsequent cloning of the genes located at the breakpoints, and potentially will provide new diagnostic tools for interphase cytogenetics *(29)*.

Furthermore, the combination of a comprehensive cytogenetic analysis with gene and protein expression profiling will provide in the near future a wealth

cytogenetic changes in cell
s, the molecular cytogenetic
yotype is surprisingly stable
esults of the SKY analysis of
the CGH analysis of primary
t the specific type of aberra-
fication of putative oncogenes
es, homogeneously staining
of chromosomal material that
ons, isochromosomes such as
nly contribute to the compre-
o to our understanding of the

e and more important for our
be studied at earlier stages of
tification of tumor-initiating
ts responsible for tumor pro-
chromosomes is challenging
and of similar size. The adap-
ge et al. *(24,27)* has proven to
ouse models *(24–28)*. Compre-
omal aberrations in the afore-
nges in the respective human
ure 1D–F displays a mouse
Y classification colors.

SKY data and to expedite the
stage-specific aberrations, a
h.gov/sky/skyweb.cgi).
omosome Aberration Project
h integrates the physical and
ne human genome *(29)*. This
C clones for the entire human
letermined by high-resolution
CAP facilitates the high-reso-
the subsequent cloning of the
y will provide new diagnostic

sive cytogenetic analysis with
ide in the near future a wealth

of information on the consequences of chromosomal aberrations in cancer, and it is hoped that this will identify entry points for the identification of new therapeutic targets and strategies.

2. Materials

2.1. Preparation of SKY Kits

1. PCR cycler.
2. Gel electrophoresis setup.
3. Speedvac.
4. Temperature-controlled microcentrifuge.
5. Primer: Telenius 6 MW(5'-CCGACTCGAGNNNNNNATGTGG-3') (100 μ*M*).
6. Nucleotides for DNA amplification: 100 m*M* dNTPs, 2 m*M* stock solution (Boehringer Mannheim, Indianapolis, IN).
7. Nucleotides for labeling:
   a. Spectrum Orange dUTP (Vysis, Downers Grove, IL); dilute 1:5 to 0.2 m*M*.
   b. Texas Red dUTP (Molecular Probes, Eugene, OR); dilute 1:5 to 0.2 m*M*.
   c. 0.1 m*M* Rhodamine 110-dUTP (Perkin-Elmer, Foster City, CA).
   d. 1 m*M* Biotin-16-dUTP (Boehringer Mannheim).
   e. 1 m*M* Digoxigenin-11-dUTP (Boehringer Mannheim).
   f. For the labeling PCR, prepare a stock solution of dNTPs with a final concentration of dATP, dCTP, and dGTP of 2 m*M*, but only 1.5 m*M* of dTTP.
8. Polymerase: native *Taq* (5 U/μL) (MBI Fermentas).
9. Buffer: 10X PCR Buffer (MBI Fermentas).
10. Human Cot-1 DNA (1 mg/mL) (Life Technologies, BRL, Grand Island, NY).
11. Salmon sperm DNA (9.7 mg/mL) (Sigma, St. Louis, MO).
12. 3 *M* Na-acetate.
13. Deionized formamide (pH 7.0).
14. Master mix: 20% dextran sulfate in 2X saline sodium citrate (SSC), pH 7.0; autoclave and store aliquots at –20°C.

2.2. Pretreatment, Denaturation, and Hybridization of Slides for SKY

1. Thermomixer or water bath.
2. Hot plate.
3. Shaker.
4. Hybridization chamber at 37°C.
5. 2X SSC.
6. RNase A (stock solution: 20 mg/mL) (Boehringer Mannheim).
7. Pepsin (stock solution: 100 mg/mL) (Sigma).
8. 0.01 *N* HCl.
9. 1X Phosphate-buffered saline (PBS).
10. 1X PBS/$MgCl_2$ (50 m*M*).
11. 1% Formaldehyde in 1X PBS/$MgCl_2$ (50 m*M*).
12. Ethanol (70, 90, 100%).
13. 70% Formamide/2X SSC (pH 7.0).

2.3. Detection

1. 50% Formamide/2X SSC (adjust to pH 7.0).
2. 1X SSC.
3. 4X SSC/Tween-20 (0.1%).
4. Blocking Solution: 3% bovine serum albumine (BSA) (Boehringer Mannheim) in 4X SSC/Tween-20; store at 4°C.
5. 1% BSA (Boehringer Mannheim) in 4X SSC/Tween-20.
6. DAPI: 80 ng/mL in 2X SCC (stock solution: 2 mg of DAPI/10 mL of sterile water).
7. Antifade: Dissolve 100 mg of 1,4-phenylenediamine in 2 mL of 1X PBS. Adjust pH with carbonate-biocarbanate buffer (pH 9.0) to 8.0, add 1X PBS to 10 mL, mix with 90 mL of 86% glycerol, aliquot and store at –20°C, and protect from light during use.
8. Mouse antidigoxin (Sigma).
9. Fluorolink-Cy5-avidin (Jackson Immuno Research, West Grove, PA).
10. Fluorolink-Cy5.5-sheep-antimouse-IgG (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.4. Image Acquisition and Analysis

1. Epifluorescence microscope equipped with a DAPI filter and SKY filter V 3.0 (Chroma Technology, Brattleboro, VT).
2. 150-W Xenon lamp (Opti-Quip, Highland Mills, NY).
3. SpectraCube™SD200, Spectral Imaging Acquisition Software, and SkyView™ software (Applied Spectral Imaging, Migdal Ha'Emek, Israel).

3. Methods

The protocols in this chapter are for SKY analysis of human chromosomes. Nevertheless, the procedure is quite similar for the mouse genome. Further information and protocols can be obtained from the following website: www.riedlab.nci.nih.gov.

3.1. Preparation of SKY Kits

3.1.1. Primary DOP-PCR

Flow-sorted chromosomes are amplified by PCR using a DOP as described by Telenius et al. *(15)*. The DNA amplification with DOPs is sequence unspecific. Therefore, employment of sterile techniques is extremely important in order to avoid contamination with genomic DNA.

Each chromosome-specific primary PCR product is labeled with a single fluorescent dye in a second DOP-PCR step for quality control purposes. Individual hybridization of all painting probes onto normal control slides should result only in specific hybridization signals for the respective pair of homologous chromosomes with low overall background. Otherwise, the primary PCR product cannot be used for the secondary and labeling DOP-PCR.

3.1.2. Secondary DOP-PCR

The primary PCR products are further amplified in a second DOP-PCR. Great precautions should be taken to avoid contamination also during this step.

1. Mix the following components for the PCR reaction: 2 µL of DNA (150–200 ng), 10 µL of PCR buffer (10X), 8 µL of $MgCl_2$ (25 m*M*), 10 µL of dNTP (2 m*M*), 65 µL of dH_2O, 4 µL of primer (100 m*M*), 1 µL of *Taq* polymerase (5 U/µL) for a total volume of 100 µL.
2. Run the following DOP-PCR program:
   a. Step 1: 94°C for 1 min.
   b. Step 2: 56°C for 1 min.
   c. Step 3: 72°C for 3 min with addition of 1 s/cycle.
   d. Step 4: Repeat **steps 1–3**, 29 times.
   e. Step 5: 72°C for 10 min.
   f. Step 6: 4°C for ∞.
3. Of the PCR product, run 2 µL on a 1% agarose gel as a quality control (intense smear between 500 bp and 2 kb).
4. Freeze DNA at –20°C.

3.1.3. DOP-PCR for Labeling

Five different fluorochromes (either directly labeled or haptenized nucleotides) are used to accomplish the differential labeling of 24 painting probes. **Table 1** was devised in order to achieve good color differences among chromosomes.

1. The setup in **Table 1** leads to 57 reactions. Label 57 autoclaved PCR tubes accordingly.
2. Mix the following components for the PCR reaction: 4 µL of DNA (400–600 ng), 10 µL of PCR buffer (10X), 8 µL of $MgCl_2$ (25 m*M*), 5 µL of dNTP (2 m*M*), dTTP (1.5 m*M*), 65 µL (for direct)/67 µL (for indirect) of dH_2O, 2 µL of primer (100 m*M*), 1 µL of *Taq* polymerase (5 U/mL); x-dUTP: 5 µL of Rhodamine 110 (0.1 m*M*), 5 µL of Spectrum Orange (0.1 m*M*), 5 µL of Texas Red (0.2 m*M*), 3 µL of biotin (1 m*M*), 3 µL of digoxigenin (1 m*M*) for a total volume of 100 µL.
3. Run the following PCR program:
   a. Step 1: 94°C for 1 min.
   b. Step 2: 56°C for 1 min.
   c. Step 3: 72°C for 3 min with addition of 1 s/cycle.
   d. Step 4: Repeat **steps 1–3**, 29 times.
   e. Step 5: 72°C for 10 min.
   f. Step 6: 4°C for ∞.
4. Run 2 µL of each DNA on a 1% agarose gel as a quality control (intense smear between 500 bp and 2 kb).
5. One SKY Kit should be precipitated according to the protocol in **Subheading 3.1.4.** and hybridized onto normal chromosomes to assess the quality. If the SKY

Table 1
Labeling Scheme

Chromosome	Rhodamine 110	Spectrum Orange	Texas Red	Cy 5 (biotin)	Cy 5.5 (digoxigenin)
1		x			x
2					x
3	x			x	x
4			x	x	
5	x	x	x		x
6			x		x
7	x			x	
8	x				
9	x	x			x
10				x	x
11		x			
12	x		x	x	x
13	x	x			
14			x		
15		x	x	x	
16	x		x	x	
17				x	
18	x	x	x		
19		x		x	
20	x	x		x	
21	x				x
22		x	x	x	x
X	x		x		
Y	x	x		x	x

Kit is of good quality, the automated classification of a normal metaphase using the SkyView software should be correct. The following points should be evaluated for quality assessment:

a. The overall painting homogeneity as well as the suppression of heterochromatin.
b. The signal-to-noise ratio: Using the software for image acquisition, the highest and lowest values for the fluorescence intensity within the image are displayed. A difference of at least 100 counts between the intensity along chromosomes and background must be achieved.
c. The color separation between chromosomes displayed in red, green or blue in the RGB image.
d. The spectra of the single dyes: The spectra of this test hybridization should be compared with and should match the reference spectra stored in the combinatorial table (ctb)-file.

Cy 5 (biotin)	Cy 5.5 (digoxigenin)
	x
	x
x	x
x	
	x
	x
x	
	x
x	x
x	x
x	
x	
x	
x	
x	
	x
x	x
x	x

n of a normal metaphase using
owing points should be evalu-

suppression of heterochromatin.
or image acquisition, the high-
nsity within the image are dis-
between the intensity along
ed.
splayed in red, green or blue in

his test hybridization should be
spectra stored in the combina-

6. If the quality of the test hybridization was good, all SKY Kits can be precipitated and stored at –20°C until further use.

3.1.4. Precipitation of SKY Kits

1. Combine 4 µL of each chromosome-painting probe (400–600 ng), 20 µL of human Cot-1 DNA and 1 µL of salmon sperm DNA in an Eppendorf tube for every SKY Kit.
2. Add 1/10 vol of 3 *M* Na-acetate and 2.5 to 3.0 times the total volume of cold 100% ethanol.
3. Vortex and precipitate at –20°C overnight or at –80°C for 30 min.
4. Centrifuge the precipitated DNA at 4°C and 11,700*g* for 30 min.
5. Remove the supernatant and dry the DNA pellet in a Speedvac for 5–10 min.
6. Add 6 µL of deionized formamide (pH 7.0), and shake in a thermomixer at 37°C until the pellet is completely dissolved (at least 1 h).
7. Add 6 µL of Master Mix, vortex, and spin briefly.
8. Store SKY Kits at –20°C until used for hybridization.

3.2. Preparation of Metaphase Chromosomes

Metaphase chromosome preparation for SKY follows standard cytogenetic protocols *(30)*. Best hybridization results are generally obtained with slides aged for 1 wk either at room temperature or in a drying oven at 37°C, if they are exposed to humidity at room temperature. Prepared slides can be stored for several years in an airtight container with desiccant at –20 or –80°C after dehydration through an ethanol series.

3.3. Pretreatment, Denaturation, and Hybridization of Slides for SKY

3.3.1. Pretreatment of Slides

1. Equilibrate slides in 2X SSC (room temperature).
2. Dilute the RNase stock 1:200 in 2X SSC, apply 120 µL per slide, and cover with a 24 × 60 mm coverslip.
3. Incubate at 37°C for 60 min.
4. Prepare 100 mL of 0.01 *N* HCl, adjust to pH 2.0, and prewarm at 37°C.
5. Remove the coverslips and wash three times for 5 min each in 2X SSC on a shaker at room temperature.
6. Pepsin treatment: Add 5–30 µL of pepsin to a Coplin jar, and then add 100 mL of prewarmed HCl, and incubate the slides at 37°C for 2 min (*see* **Note 1**).
7. Wash twice for 5 min each in 1X PBS at room temperature, shaking.
8. Wash once for 5 min in 1X PBS/$MgCl_2$.
9. Incubate the slides for 10 min at room temperature in 1% formaldehyde in 1X PBS/$MgCl_2$ for postfixation.
10. Wash again one time for 5 min in 1X PBS at room temperature, shaking.
11. Dehydrate the slides in 70, 90, and 100% ethanol for 3 min each.
12. Let the slides air-dry (*see* **Note 1**).

3.3.2. Denaturation of SKY Kit

1. Prewarm SKY Kits at 37°C for 30 min.
2. Denature SKY Kits at 80°C for 5 min in a thermomixer or water bath,
3. Before applying to the slide, allow the SKY Kit to preanneal at 37°C for 1 to 2 h.

3.3.3. Slide Denaturation

1. Apply 120 µL of 70% formamide/2X SSC to a 24 × 60 mm coverslip and touch slide to coverslip.
2. Denature the slides at 75°C on a slide warmer for 1 min, 30 s. Denaturation of slides can also be performed by preheating 70% formamide/2X SSC in a Coplin jar in a water bath to 72°C. This is especially applicable for G-banded slides, for which denaturation times are shorter (10–30 s).
3. Shake off the coverslips and immediately place the slides in freshly prepared 70% ethanol (precooled to 0°C) for 3 min, followed by 3 min in 90% and 100% ethanol each.
4. Let the slides air-dry.

3.3.4. Hybridization

1. After preannealing, add the SKY Kit to the preselected hybridization area on the denaturated slides and cover with an 18-mm^2 coverslip.
2. Seal the coverslips with rubber cement and incubate in a hybridization chamber at 37°C for 48 h. Drying out of the SKY Kit during the hybridization time should be avoided.

3.4. Detection

1. Prepare solutions (formamide/SSC, 1X SSC, 4X SSC/Tween-20) and prewarm at 45°C for 30 min before starting the detection.
2. After the hybridization time, carefully remove the rubber cement and dip the slides in formamide/SSC until the coverslips slide off (*see* **Note 2**).
3. Wash the slides three times for 5 min each in formamide/SSC, shaking.
4. Wash the slides three times for 5 min each in 1X SSC, shaking.
5. Dip the slides in 4X SSC/Tween-20.
6. Incubate the slides with blocking solution (120 µL/slide, covered with a 24 × 60 mm coverslip) in a hybridization chamber at 37°C for 30 min.
7. Spin all the fluorescent dyes for 3 min at 13,000 rpm.
8. Dip the slides in 4X SSC/Tween-20.
9. Add 120 µL of antibody solution containing mouse antidigoxin (1:200 dilution in 1% BSA) per 24 × 60 mm coverslip, touch the slide to the coverslip, and incubate in a hybridization chamber for 1 h at 37°C.
10. Wash the slides three times for 5 min each in 4X SSC/Tween-20, shaking.
11. Add 120 µL of antibody solution containing avidin-Cy5 and Cy5.5 antimouse (1:200 dilution in 1% BSA each) per coverslip (24 × 60 mm), touch the slide to the coverslip, and incubate in a hybridization chamber for 1 h at 37°C.

12. Wash the slides three times for 5 min each in 4X SSC/Tween-20, shaking.
13. Stain with DAPI for 5 min in a light-protected Coplin jar.
14. Wash for 5 min in 2X SSC, shaking.
15. Dehydrate the slides in an ethanol series (70, 90, 100%) for 3 min each.
16. Let the slides air-dry in the dark.
17. When the slides are completely dry, apply 30 µL of antifade, cover with 24 × 60 mm coverslips, and store in the dark at 4°C until image acquisition.

3.5. Image Acquisition and Analysis

For each metaphase, a spectral image and the corresponding DAPI image is acquired using an epifluorescence microscope connected to the SpectraCube (Applied Spectral Imaging; a combination of a Sagnac-Interferometer and a CCD-camera). For the spectral image, a custom-designed SKY filter (Chroma) is employed; the DAPI image is acquired using the TR1-filter (Chroma). The subsequently inverted DAPI-image gives a chromosomal banding pattern comparable with the one obtained by G-banding (**Fig. 1B,E**). During image acquisition, heat protection filters should normally be placed into the light pass but can be removed if the intensity of the fluorescent dyes with emission in the far red range (Cy5 and Cy5.5) is weak.

For image analysis, the spectral image is first displayed in RGB (red-green-blue) colors. This allows for the evaluation of hybridization quality (**Fig. 1A,C**). Using the SkyView software, both the spectral and the DAPI image are then analyzed simultaneously. Through correlation of the spectral information with the labeling scheme and the reference spectra of the five fluorescent dyes (stored in a ctb–file) a specific pseudocolor is assigned to each image point. Thus, all material belonging to the same chromosome will be displayed in the same pseudocolor, and chromosomal aberrations will be easily visible (**Fig. 1C, F**).

4. Notes

1. Pretreatment with pepsin to remove residual cytoplasm is a crucial step because overtreatment with pepsin leads to reduced signal intensity and impaired chromosome morphology and therefore compromises SKY results. Pepsin concentration and time must therefore be adjusted according to the amount of cytoplasm; that is, use low concentrations of pepsin (5–10 µL; 2 min) if there is little cytoplasm, and 20–30 µL, 5 min, for cells with high amounts of cytoplasm. Cytoplasm is visible as opaque material around the metaphase chromosomes. If no cytoplasm is present, pepsin treatment may not be necessary at all.
2. During the detection avoid exposure to light as much as possible and avoid air-drying of the slides between the different steps. Slides should be handled carefully in order to avoid scratching the surfaces.

Acknowledgments

We gratefully acknowledge Prof. M. A. Ferguson-Smith and Dr. J. Wienberg for providing high-quality flow-sorted chromosomes. E. H. received a postdoctoral fellowship from the Deutsche Krebshilfe.

References

1. Caspersson, T., Zech, L., and Johansson, C. (1970) Differential banding of alkylating fluorochromes in human chromosomes. *Exp. Cell Res.* **60,** 315–319.
2. Thompson, F. H. (1997) Cytogenetic methods and findings in human solid tumors, in *The AGT Cytogenetics Laboratory Manual*, 3rd ed., (Barch, M. J., Knutsen, T., and Spurbeck, J., eds.), Lippincott-Raven, Philadelphia, PA, pp. 375–30.
3. Heim, S. and Mitelman, F. (eds.) (1995) *Cancer Cytogenetics,* 2nd ed., Wiley-Liss, New York.
4. Mertens, F., Johansson, B., Höglund, M., and Mitelman, F. (1997) Chromosomal imbalance maps of malignant solid tumors: a cytogenetic survey of 3185 neoplasms. *Cancer Res.* **57,** 2765–2780.
5. Cremer, T., Lichter, P., Borden, J., Ward, D. C., and Manuelidis, L. (1988) Detection of chromosome aberrations in metaphase and interphase tumor cells by in situ hybridization using chromosome-specific library probes. *Hum. Genet.* **80,** 235–246.
6. Pinkel, D., Landegent, J., Collins, C., Fuscoe, J., Segraves, R., Lucas, J., and Gray, J. W. (1988) Fluorescence in situ hybridization with human chromosome specific libraries: detection of trisomy 21 and translocation of chromosome 4. *Proc. Natl. Acad. Sci. USA* **85,** 9138–9142.
7. Kallioniemi, A., Kallioniemi, O.-P., Sudar, D., Rutovitz, D., Gray, J. W., Waldman, F., and Pinkel, D. (1992) Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. *Science* **258,** 818–821.
8. Romana, S. P., Le Coniat, M., and Berger, R. (1994) t(12;21): A new recurrent translocation in acute lymphoblastic leukemia. *Genes Chromosomes Cancer* **9,** 186–191.
9. Shurtleff, S. A., Buijs, A., Behm, F. G., Rubnitz, J. E., Raimondi, S. C., Hancock, M. L., et al. (1995) TEL/AML1 fusion resulting from a cryptic t(12;21) is the most common genetic lesion in pediatric ALL and defines a subgroup of patients with an excellent prognosis. *Leukemia* **9,** 1985–1989.
10. Forozan, F., Karhu, R., Kononen, J., Kallioniemi, A., and Kallioniemi, O. P. (1997) Genome screening by comparative genomic hybridization. *Trends Genet.* **13,** 405–409.
11. Ried, T., Heselmeyer-Haddad, K., Blegen, H., Schröck, E., and Auer, G. (1999) Genomic changes defining the genesis, progression, and malignancy potential in solid human tumors: a phenotype/genotype correlation. *Genes Chromosomes Cancer* **25,** 195–204.
12. Speicher, M., Ballard, S. G., and Ward, D. C. (1996) Karyotyping human chromosomes by combinatorial multi-fluor FISH. *Nat. Genet.* **12,** 368–375.
13. Schröck, E., du Manoir, S., Veldman, T., Schoell, B., Wienberg, J., Ferguson-Smith, M. A., et al. (1996) Multicolor Spectral Karyotyping of human chromosomes. *Science* **273,** 494–497.

-Smith and Dr. J. Wienberg
somes. E. H. received a
lfe.

Differential banding of alky-
Cell Res. **60,** 315–319.
ndings in human solid tumors,
l., (Barch, M. J., Knutsen, T.,
hia, PA, pp. 375–30.
Cytogenetics, 2nd ed., Wiley-

man, F. (1997) Chromosomal
genetic survey of 3185 neo-

Manuelidis, L. (1988) Detection
se tumor cells by in situ hybrid-
m. Genet. **80,** 235–246.
raves, R., Lucas, J., and Gray,
human chromosome specific
of chromosome 4. *Proc. Natl.*

Rutovitz, D., Gray, J. W.,
e genomic hybridization for
ence **258,** 818–821.
(12;21): A new recurrent trans-
omosomes Cancer **9,** 186–191.
E., Raimondi, S. C., Hancock,
from a cryptic t(12;21) is the
defines a subgroup of patients
39.
., A., and Kallioniemi, O. P.
hybridization. *Trends Genet.*

röck, E., and Auer, G. (1999)
n, and malignancy potential in
on. *Genes Chromosomes Can-*

96) Karyotyping human chro-
Genet. **12,** 368–375.
, B., Wienberg, J., Ferguson-
aryotyping of human chromo-

14. Garini, Y., Macville, M., du Manoir, S., Buckwald, R.A., Lavi, M., Katzir, N., et al. (1996) Spectral karyotyping. *Bioimaging* **4,** 65–72.
15. Telenius, H., Pelear, A. H., Tunnacliffe, A., Carter, N. P., Behmel, A., Ferguson-Smith, M. A., et al. (1992) Cytogenetic analysis by chromosome painting using DOP-PCR amplified flow-sorted chromosomes. *Genes Chromosomes Cancer* **4,** 257–263.
16. Knutsen, T. and Ried, T. (2000) SKY: a comprehensive diagnostic and research tool: a review of the first 300 published cases. *J. Assoc. Genet. Technol.* **26,** 3–15.
17. Adeyinka, A., Kytola, S., Mertens, F., Pandis, N., and Larsson, C. (2000) Spectral karyotyping and chromosome banding studies of primary breast carcinomas and their lymph node metastases. *Int. J. Mol. Med.* **5,** 235–240.
18. Wong, N., Lai, P., Pang, E., Wai-Tong Leung, T., Wan-Yee Lau, J., and Johnson, P. J. (2000) A comprehensive karyotypic study on human hepatocellular carcinoma by spectral karyotyping. *Hepatology* **32,** 1060–1068.
19. Phillips, J. L., Ghadimi, B. M., Wangsa, D., Padilla-Nash, H., Worrell, R., Hewitt, S., Linehan, W. M., et al. (2001) Cytogenetic characterization of early and late renal cell carcinomas in von Hippel-Lindau (VHL) disease. *Genes Chromosomes Cancer* **31,** 1–9.
20. Macville, M., Schröck, E., Padilla-Nash, H., Keck, C., Ghadimi, B. M., Zimonjic, D., et al. (1999) Comprehensive and definitive molecular cytogenetic characterization of HeLa cells by spectral karyotyping. *Cancer Res.* **59,** 141–150.
21. Ghadimi, B. M., Schröck, E., Walker, R. L., Wangsa, D., Jauho, A., Meltzer, P. S., and Ried, T. (1999) Specific chromosomal aberrations and amplification of the *AIB1* Nuclear Receptor Coactivator Gene in pancreatic carcinomas. *Am. J. Pathol.* **154,** 525–536.
22. Ghadimi, B. M., Sackett, D. L., Difilippantonio, M. J., Schröck, E., Neumann, T., Jauho, A., et al. (2000) Centrosome amplification and instability occurs exclusively in aneuploid, but not in diploid colorectal cancer cell lines, and correlates with numerical chromosomal aberrations. *Genes Chromosomes Cancer* **27,** 183–190.
23. Padilla-Nash, H. M., Heselmeyer-Haddad, K., Wangsa, D., Zhang, H., Ghadimi, B. M., Macville, M., et al. (2001) Jumping translocations (JT) are common in solid tumor cell lines and result in recurrent fusions of whole chromosome arms. *Genes Chromosomes Cancer* **30,** 349–363.
24. Liyanage, M., Coleman, A., du Manoir, S., Veldman, T., McCormack, S., Dickson, R. B., et al. (1996) Multicolour spectral karyotyping of mouse chromosomes. *Nat. Genet.* **14,** 312–315.
25. Coleman, A. E., Schrock, E., Weaver, Z., du Manoir, S., Yang, F., Ferguson-Smith, M. A., et al. (1997) Previously hidden chromosome aberrations in T(12;15)-positive BALB/c plasmacytomas uncovered by multicolor spectral karyotyping. *Cancer Res.* **57,** 4585–4592.
26. Weaver, Z. A., McCormack, S. J., Liyanage, M., du Manoir, S., Coleman, A., Schröck, E., et al. (1999) A recurring pattern of chromosomal aberrations in mammary gland tumors of MMTV-*cmyc* transgenic mice. *Genes Chromosomes Cancer* **25,** 251–260.

27. Liyanage, M., Weaver, Z., Barlow, C., Coleman, A., Pankratz, D. G., Anderson, S., et al. (2000) Abnormal rearrangement within the a/d T-cell receptor locus in lymphomas from Atm-deficient mice. *Blood* **96,** 1940–1946.
28. Difilippantonio, M. J., Zhu, J., Chen, H. T., Meffre, E., Nussenzweig, M. C., Max, E. E., et al. (2000) DNA repair protein Ku80 suppresses chromosomal aberrations and malignant transformation. *Nature* **404,** 510–514.
29. Kirsch, I. R., Green, E. D., Yonescu, R., Strausberg, R., Carter, N., Bentley, D., et al. (2000) A systematic, high-resolution linkage of the cytogenetic and physical maps of the human genome. *Nat. Genet.* **24,** 339–340.
30. Barch, M. J., Knutsen, T., and Spurbeck, J. L. (eds.) (1997) *The AGT Cytogenetics Laboratory Manual*, 3rd ed., Lippincott-Raven, Philadelphia, PA.